Identification and functional characterization of an RNA binding protein involved in defense signaling pathway and cell death in rice.

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Abstract

U-box containing ubiquitin E3 ligase proteins play key roles in the regulation of diverse developmental processes such as cell death, defense responses, and flowering in monocots and dicots. We have previously demonstrated that *SP*OTTED *L*EAF 11 (SPL11), a rice U-box E3 ubiquitin ligase, is essential for the fine tuning of programmed cell death (PCD) and flowering in rice. Loss of function *spl11* gamma-ray mutant showed lesion mimics, enhanced resistance to two different rice pathogens and delayed flowering under the long day conditions (Zeng et al., 2004; Vega-Sanchez et al., 2008). To elucidate the molecular and biochemical mechanisms by which SPL11 controls PCD and/or flowering time, yeast two-hybrid (Y2H) screens were performed using SPL11 as the bait, and eight proteins were identified as putative *SP*L11 *in*teractors (SPINs). Among them, SPIN1, a signal *t*ransduction and *a*ctivation of *R*NA (STAR), interacts with SPL11 in the nucleus and represses flowering via transcriptional perturbation of *h*eading *d*ate *3a* (*Hd3a*), an ortholog of *FLOWERING LOCUS T* (*FT*) in *Arabidopsis* (Vega-Sanchez et al., 2008). Interestingly, another RNA-binding protein, *R*NA *b*inding interactor of SPIN1 (RBS1), was identified through additional Y2H screens using SPIN1 as the bait. Transient expressions of RBS1 in rice protoplasts and leaves of *Nicotiana benthamiana* showed abrupt host cell death in both systems. In addition, over-expression of *RBS1* in rice resulted in lesion mimicry and dwarfism in T1 generation. These results suggest that SPL11 might regulate program cell death through SPIN1/RBS1 protein complex.

Introduction

Plant diseases cause significant yield loss of many crops over the world and elucidating the molecular mechanism of host resistance to pathogens can help us design new approaches to reduce the loss of economically important crops. Even though rice has been a model plant for cereal crops, the defense mechanism to two important pathogens, *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), has not been clearly understood yet. It has been known that lesion mimic formation in plants can activate defense responses even in the absence of pathogens (Dangl et al., 1996; Lorrain et al., 2003). These lesions are similar in appearance to necrotic lesion or hypersensitive reaction (HR) lesion caused by pathogen attack and this type of mutants was termed "lesion mimic mutants (LMM)". LMM are one of the valuable resources to study both programmed cell death (PCD) and defense signaling in plants. Although many lesion mimic genes have been cloned in plants, the molecular mechanism on how they control PCD and what their relationship with defense responses is has not been well elucidated. RNA binding proteins are known to be involved in the process and transport of pri-mRNA, translation and degradation of various types of RNA (Alba and Pages, 1998). Although there have been many studies of RNA binding proteins in vertebrates and yeast, much less is known about their functions in plants. So far, only two RNA-binding proteins have shown their roles in PCD in plants. The eukaryotic initiation factor 5A (eIF-5A) positively controls in senescence and cell death by fumonisin B1 and is involved in cell growth and development in *Arabidopsis* (Thompson et al., 2004; Feng et al., 2007). Similarly, over-expression of the RNA-binding UBA2 gene leads to a leaf yellowing/cell death-like phenotype in *Arabidopsis* plants, indicating its role as a positive regulator of senescence and cell death in *Arabidopsis* (Kim et al., 2008). In this study, we aimed to identify the function of the *Rbs1* gene in the *Spl11*-mediated PCD and defense response in rice. Both over-expression and knock-down transgenic lines of *Rbs1* have been generated. Obviously cell death and dwarfing phenotypes were observed in the *Rbs1* overexpressing transgenic plants, indicating a possible role of *Rbs1* in the PCD regulation. Detailed molecular and phenotypic analyses of these plants are being performed. We hypothesize that SPL11 interacts indirectly with RBS1 via SPIN1 and the protein complex negatively regulates PCD in rice.

Materials and Methods

Plant Growth: Rice seeds were germinated on 1/2 MS for three days and transferred to soil in a Conviron growth chamber (Conviron CMP 4030, Manitoba) at 85% RH with a day/night regime of 12 hr light (500 µmol photons m-2 sec-1) at 26 º C followed by 12 hr dark at 20 º C.
Cell Death Analysis of *Rbs1* in Rice Protoplasts: RNAi constructs containing the candidate gene fragments and the construct containing the beta-glucuronidase (GUS) gene under the control of the maize ubiquitin promoter were transiently expressed in rice protoplasts as described (Chen et al., 2006). The assay of GUS activity was carried out according to the protocol described by Chen et al. (2006).
Subcellular localization experiments: The *DsRed-Rbs1* translational fusion was obtained by cloning an *Rbs1* coding sequence PCR fragment into the pGDR vector (Goodin et al., 2002) to make pGRbs1-Red. The *GFP-Spin1* translational fusion was made in the pGDG (pGSpin1-GFP). The pGRbs1-Red and pGSpin1-GFP constructs were transformed into *Agrobacterium tumefasciens* strain GV3101 and used to agroinfiltrate 4 week-old *N. benthamiana* plants as described in (Goodin et al., 2002). After the infiltration of pGRbs1-Red and pGSpin1-GFP constructs, infiltrated leaves were observed under a fluorescent microscope to observe the localization of DsRed-RBS1 and/or GFP-SPIN1 fusion proteins. For sub-cellular localization in rice cells, the same constructs used for agroinfiltration were used to transform rice seedling protoplasts. The protocols for rice protoplast isolation and PEG-mediated transformation were previously described in Chen et al. (2006).
RNA binding assay: Beads containing polyadenylic, polycytidylic, polyuridylic and polyguanylic ribohomopolymers were purchased from Sigma. Approximately 30ng of recombinant GST-SPIN1-HA was incubated with the beads in 500µl of KHN buffer (150mM KCl, 20mM HEPES pH7.9, 0.01% Nonidet P-40, complete protease inhibitors) under rotation for 10 min. Beads were washed in KHN buffer 3 times and proteins retained in the beads were identified by western blot analysis using either anti-HA (Roche) or anti-GST (Invitrogen) antibodies.
Agroinfiltration experiments: The *Rbs1* and *Spin1* coding sequences were PCR-amplified and cloned into the pGD vector (Goodin et al., 2002). The pGWtsE plasmid was described elsewhere (Ham et al., 2006). Constructs were transformed into *A. tumefasciens* strain GV3101 and used to infiltrate *N. benthamiana* plants as previously described in Goodin et al. (2002).
Yeast two-hybrid screen: The ProQuest yeast two-hybrid system (Invitrogen) was used to screen for SPIN1-interacting proteins following the manufacturer's protocol. A full-length *Spin1* cDNA was used as the bait by cloning it into the pDBleu vector. A rice cDNA library made with the pPC86 vector was used as the prey. Putative interacting candidate clones were sequenced at the Plant-Microbe Genomics Facility (PMGF) of the Ohio State University.

Results

Identification of *Rbs1* by Y2H with *Spin1* full length cDNA: To identify interacting proteins with SPIN1 in rice, a yeast two-hybrid screen was performed using the full length *Spin1* cDNA as the bait and a rice cDNA library as the prey (Figure 1A). Only two clones were able to interact with SPIN1 reproducibly after two rounds of screening using half a million-yeast colonies. The two cDNAs have identical sequence corresponding to the full length cDNA of Os11g14430. Protein motif analysis using Pfam and BlastX programs revealed the deduced amino acid sequence of Os11g14430 contains three RNA Recognition Motifs (RRMs), suggesting it is a putative RNA-binding protein. Then, the candidate gene is named as *Rbs1*, for RNA-binding and SPIN1-interacting 1.

Since it has been known that the Y2H screening can give false positive results and also in order to determine whether RBS1 can interact with SPIN1 *in vivo*, a protoplast-based two hybrid (P2H) screening experiment was conducted. The full length cDNAs of *Spin1* and *Rbs1* were cloned into the two vectors containing yeast GAL4 DNA biding (DB) and activation (AD) domains, respectively, and the interaction between the two proteins were monitored by the activation of a GUS (β-glucuronidase) reporter gene driven by the GAL promoter sequence. As shown in Figure 1B, only when both the *Rbs1* and *Spin1* constructs were co-transfected into rice protoplasts, GUS activity was increased compared to the control and the transfection of each construct. JUN and FOS were used as the positive controls as they are known to interact with each other *in vivo*.

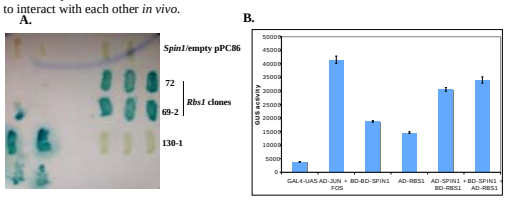

Figure 1. RBS1 interacts with SPIN1 in yeast and in rice protoplasts. **A.** Identification of *Rbs1* by yeast two-hybrid screen of a rice cDNA library using full length *Spin1* as the bait. 72 and 69-2 are independent yeast clones containing the pDBleu-*Spin1* and pPC86-*Rbs1* constructs. Positive clones were identified by an X-GAL assay. **B.** Protoplast two-hybrid (P2H) assay confirming the *in vivo* interaction between SPIN1 and RBS1. GUS activity was measured after transfection of rice protoplasts with GAL4 activation (AD) and DNA binding (BD) fusions to SPIN1 and/or RBS1. The interaction between AD-JUN and BD-FOS is shown as a positive control for the P2H assay. Error bars: +/- standard deviation. The assay was repeated twice with similar results.

Co-localization of RBS1 and SPIN1: Since the *Rbs1* gene encodes a putative RNA-binding protein and SPIN, its interacting protein, is a nuclear-targeted protein, it is hypothesized that RBS1 should be localized in the nucleus. The full cDNA of *Rbs1* and *Spin1* were fused with DsRed and GFP protein, respectively. Then both constructs were agroinfiltrated into *N. benthamiana* plants and co-transfected into rice protoplasts, respectively. As shown in Figure 2, both RBS1-DsRed and SPIN1-GFP were co-localized in the nucleus of *N. benthamiana* cells and rice protoplasts.

Results (cont.)

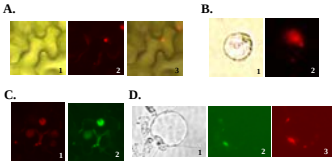

Figure 2. Subcellular localization of DsRed-RBS1. **A.** DsRed-RBS1 localizes to the nucleus in *Nicotiana benthamiana* plants; 1: bright field; 2: red fluorescence; 3: 1 and 2 merged. **B.** DsRed-RBS1 localizes to the nucleus in rice protoplasts; 1: bright field; 2: red fluorescence. **C.** GFP-SPIN1 and DsRed-RBS1 co-localize in the nucleus of *N. benthamiana* cells; 1: red fluorescence; 2: green fluorescence. **D.** GFP-SPIN1 and DsRed-RBS1 co-localize in the nucleus of rice protoplasts; 1: bright field; 2: green fluorescence; 3: red fluorescence.

RNA Binding Assay: Protein motif analysis suggested that RBS1 is a putative RNA-binding protein. To confirm this *in vitro*, the full length cDNA of *Rbs1* was cloned into the protein expression vector pGex-6p-1and was *in vitro* translated and labeled with biotin using the rabbit reticulocyte lysate system. The translated RBS1 protein was incubated with ribohomopolymer beads containing poly A, poly U, poly C and poly G RNA molecules and detected with streptavidin-HRP substrate in western blotting. As shown in Figure 3, RBS1 binded to polyA, poly U and poly G, but not to poly C RNA oligos, suggesting that RBS1 protein has RNA binding activity *in vitro*.

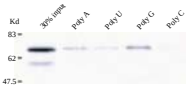

Figure 3. RNA binding assay for RBS1. *In vitro* translated RBS1 protein was incubated with ribohomo-polymers to test for *in vitro* RNA binding activity. RBS1 binds preferentially to Poly G RNA.

Y2H and Bimolecular Florescence Complementation (BiFC) of RBS1 with SPL11: Since SPL11 interacts with SPIN1 in yeast and in vitro (Vega-Sanchez et al., 2008), it was speculated that there might be interaction between RBS1 and SPL11. To confirm this, Y2H, BiFC and P2H experiments were carried out. No interaction between these two proteins was detected in these assays (data not shown).

Cell Death Assay in Rice Protoplasts and *N. benthamiana*: To test whether over-expression of *Rbs1* causes cell death in plants, the full length cDNA of *Rbs1* was agro-infiltrated into *N. benthamiana* plants. As shown in Figure 4A and B, a patch of dead cell showed up in the area where *Rbs1* was infiltrated while no cell death was observed with an empty vector control. The intensity of cell death by over-expression of *Rbs1* was not as strong as the positive control effector protein WtsE from *Pantoea stewartii*. However, 32 out of 35 35S:*Rbs1* infiltrated plants showed cell death phenotype, suggesting this phenotype is reliable. Also when *Rbs1* was over-expressed in rice protoplasts, it caused the cell death compared to the vector control (Figure 4C.), complementing the experiment in *N. benthamiana*. Recently, we generated the *Rbs1* over-expression transgenic plants

Dwarfism and Cell Death in Rbs1 Over-expression T1 lines: More than 10 independently transformed overexpression transgenic lines of the *Rbs1* gene were obtained. Obvious lesion mimics and dwarfism in the T1 lines were observed (Figure 5.) Lesions appeared as water soaking spots with chlorosis initially and turned to dark brown color at later stages. This phenotype will be confirmed in the next generation.

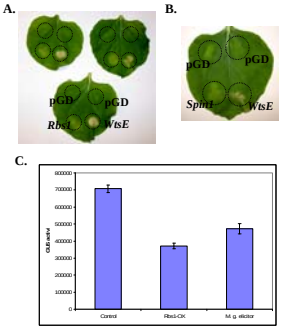

Figure 4. Overexpression of *Rbs1* in *N. benthamiana* and rice protoplasts causes cell death. **A.** Agroinfiltration of *35S:Rbs1* in *N. benthamiana.* WtsE is a bacterial effector that induces a strong cell death and was used as the positive control; pGD: empty vector control. **B**. Agroinfiltration of 35S:*Spin1* in *N.benthamiana*. Notice the lack of cell death. **C.** GUS activity in rice protoplasts transfected with pUbi-*Rbs1* (a construct overexpressing *Rbs1*), empty vector (control) or treated with the *M. oryzae* cell wall elicitor. The *M. oyzae* elicitor was used as a positive control for cell death in rice.

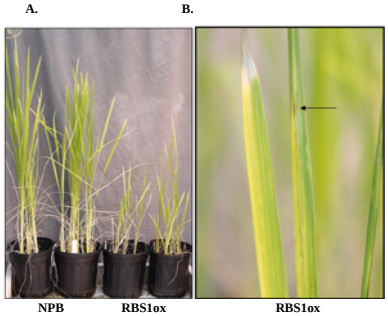

Figure 5. Overexpression of *Rbs1* causes dwarfism and cell death in stable transgenic lines.
A. Dwarfism in *Rbs1* over-expression T1 lines
B. Cell death in *Rbs1* over-expression T1 lines. Arrow indicates cell death region in the leaf. NPB: Nipponbare (recipient cultivar). RBS1ox: RBS1 over-expression lines

Conclusions and Discussion

In this study, we identified RBS1 in the Y2H screen using SPIN1 as the bait and showed that RBS1 have RNA binding activity in vitro. Transient expression of *Rbs1* in rice protoplasts by transfection and in *N. benthamiana* by agro-infiltration caused cell death. Furthermore, stable transgenic lines which over-express the full length cDNA of *Rbs1* showed dwarfism and cell death phenotypes. Evaluations of the resistant levels to rice blast and bacterial blight pathogens are in progress. It is puzzling that over-expression or silencing of *Spin1* does not show any cell death phenotype but the *Rbs1* over-expression does. What is the relationship between *Spl11* and *Rbs1*? We speculate that even though SPL11 doesn't interact with RBS1 directly, SPL11 and RBS1 could form a protein complex via the SPIN1 protein. For the future experiments, we will focus on these two objectives: 1) analyzing the cell death and resistance phenotypes of *Rbs1* over-expression and RNAi lines, and 2) identifying the link between *Spl11* and *Rbs1* in PCD and defense response control. Functional analysis of the novel rice RNA binding protein will provide the first direct evidence for RNA binding protein's involvement in both PCD and defense responses.

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